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Chromosome Association of Minichromosome Maintenance Proteins in *Drosophila* Mitotic Cycles

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Abstract. Minichromosome maintenance (MCM) proteins are essential DNA replication factors conserved among eukaryotes. MCMs cycle between chromatin bound and dissociated states during each cell cycle. Their absence on chromatin is thought to contribute to the inability of a G₂ nucleus to replicate DNA. Passage through mitosis restores the ability of MCMs to bind chromatin and the ability to replicate DNA. In *Drosophila* early embryonic cell cycles, which lack a G₁ phase, MCMs reassociate with condensed chromosomes toward the end of mitosis. To explore the coupling between mitosis and MCM–chromatin interaction, we tested whether this reassociation requires mitotic degradation

of cyclins. Arrest of mitosis by induced expression of nondegradable forms of cyclins A and/or B showed that reassociation of MCMs to chromatin requires cyclin A destruction but not cyclin B destruction. In contrast to the earlier mitoses, mitosis 16 (M16) is followed by G₁, and MCMs do not reassociate with chromatin at the end of M16. *dacapo* mutant embryos lack an inhibitor of cyclin E, do not enter G₁ quiescence after M16, and show mitotic reassociation of MCM proteins. We propose that cyclin E, inhibited by Dacapo in M16, promotes chromosome binding of MCMs. We suggest that cyclins have both positive and negative roles in controlling MCM–chromatin association.

THE competence of a nucleus to replicate its DNA oscillates during the cell cycle. For example, a G₁ nucleus is able to replicate DNA given the appropriate signals, while a G₂ nucleus is unable to do so given the same signals (Rao and Johnson, 1970; reviewed in Heichman and Roberts, 1994; Coverley and Laskey, 1994). Because a G₁ state arises from a G₂ state upon passage through mitosis, mitosis restores the competence to replicate DNA. Restoration of replication competence at mitosis is thought to be marked and perhaps caused by association of minichromosome maintenance (MCM)¹ proteins with chromatin (Todorov et al., 1994; Kimura et al., 1994; Chong et al., 1995; Madine et al., 1995b; Todorov et al., 1995; Coue et al., 1996; Schulte et al., 1996). MCMs are evolutionarily conserved replication factors that function in an early step of DNA replication (reviewed in Tye, 1994, and Chong et al., 1996; see also Hennessy et al., 1991; Thommes et al., 1992; Yan et al., 1993; Miyake et al., 1993). In a premitotic nucleus, MCMs are not associated with chromatin; as vertebrate nuclei progress through mitosis, association of

MCMs with chromosomes occurs in parallel with mitotic restoration of replication competence (Todorov et al., 1994; Schulte et al., 1996; Coue et al., 1996). To understand how passage through mitosis is coordinated with DNA replication, we have asked what triggers chromosome association of MCMs during mitosis.

Studies in a variety of experimental systems suggest that the key mitotic event that restores the ability to replicate DNA is the loss of cyclins or associated activities. In *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Drosophila*, and vertebrate cultured cells, experimental removal of mitotic cyclins or cdks allow DNA replication in a G₂ nucleus (Broek et al., 1991; Hayles et al., 1994; Dahmann et al., 1995; Sauer et al., 1995; Hayashi, 1996; Coverley et al., 1996). These data demonstrate that mitotic cyclin:cdks prevent DNA replication in G₂, and they suggest that loss of cyclin:cdks during mitosis restores the competence to replicate DNA. The mechanisms by which cyclin:cdks prevent rereplication in G₂ may include preventing MCMs from binding to chromatin for the following reasons: Cyclin:cdks can prevent an assembly of proteins called the “prereplicative complex” on origins of DNA replication in *S. cerevisiae* (Dahmann et al., 1995; Piatti et al., 1996); prereplicative complexes are thought to contain MCMs (reviewed in Nasmyth, 1996; Diffley, 1997). Additionally, an inhibitor of MCM function is present in *Xenopus* mitotic extracts and can be removed with Suc1 beads, a procedure that depletes cdks (Mahbubani et al., 1997).

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1. *Abbreviations used in this paper:* aa, amino acid; M16, mitosis 16; MCM, minichromosome maintenance (protein); NPC, nuclear pore complex; RLF, replication licensing factor; rt, room temperature.

These data led us to ask if the mitotic loss of cyclin:cdk activity couples passage through mitosis to chromosome association of MCMs. Mitotic inactivation of cyclin:cdks occurs by proteolytic degradation of the cyclin subunits. In the *Drosophila* embryo cyclins A and B provide essential, nonredundant, mitotic functions (Knoblich and Lehner, 1993). We therefore analyzed the consequence of preventing degradation of cyclins A and B on *Drosophila* MCMs. We demonstrate here that mitotic association of MCMs with chromosomes occurs when degradation of cyclin B was prevented but not when degradation of cyclin A was prevented. We infer from this data that mitotic degradation of cyclin A permits MCMs to associate with chromosomes. After the 16th mitosis (M16), epidermal cells of the embryo withdraw from the cell cycle and enter a prolonged gap phase (G₁17). During M16, MCMs failed to associate with chromosomes despite the absence of cyclin A. The withdrawal of epidermal cells from the cell cycle depends on the turn-on of Dacapo, a cdk inhibitor (cki) specific for cyclin E:cdk2 (de Nooij et al., 1997; Lane et al., 1997). In *dacapo* mutants, chromosome association of MCMs is detected in M16, suggesting that Dap inhibits this association normally. We infer that in addition to destruction of cyclin A, MCM-chromosome association requires an activity that can be inhibited by Dacapo.

Materials and Methods

Fixation and Antibody Staining Procedures

For antibody staining, embryos were fixed and stained with purified primary antibodies, and diluted 1:50–1:500 using standard procedures. Specificity of rabbit polyclonal antibodies against each MCM has been described previously (Su et al., 1996). Anti- β -tubulin antibody (Amersham, Arlington Heights, IL) was used at 1:100 dilution. Primary antibodies were detected either with fluorescent secondary antibodies (Jackson ImmunoResearch Laboratories; West Grove, PA) or by histochemical staining using the peroxidase reaction. DNA was stained with 10 mg/ml bisbenzamid (Hoechst 33258). WGA (Molecular Probes, Inc., Eugene, OR) was used at 500 ng/ml.

Heat Induction

Stable versions of mitotic cyclins were constructed essentially as described previously (Sigrist et al., 1995). Briefly, stable cyclin A lacks amino acids (aa) 2–170. Stable cyclin B lacks aa 3–47 and has a glycine as the second aa residue, before aa 48 from the wild-type sequence (Sprenger et al., 1997). Both cyclins were produced from hsp70 promoter and carried as homozygous lines. w⁶⁷, the isogenic stock used for carrying the transgenes, was used as a control in all heat shock experiments. *Drosophila* embryos were collected for 2 h and aged for either 2 h at room temperature (rt; to reach cycle 14) or for 5 h at rt (to reach cycle 16) before heat shock. Embryos were heat shocked by floating grape juice–agar plates on water at 37° for 30 min. After recovery at rt for 2 h, embryos were fixed for 20 min in a two-phase mixture of heptane and fix (PBS + 10% formaldehyde) according to standard procedures, and were processed for antibody staining as described above. The level of induced stable cyclins is about two- to three fold over that of endogenous cyclins. Cell cycle and developmental stages of embryos were confirmed by the morphology of embryos and the density of cells.

dacapo Mutants

Embryos were collected from heterozygous *dap^{3x41}* parents for 1 h and aged at 25° for 5.5 h to reach cycle 16 before fixing. This is a mutant resulting from imprecise excision of the transposon from the *dap³* line (de Nooij et al., 1997). A Cyo wg-LacZ balancer was used to identify the genotype to embryos by immunostaining for β -galactosidase (Amersham antibodies). Homozygotes were identified by the lack of β -galactosidase staining.

Results

MCMs Associate with Chromosomes as Mitosis Is Completed

Identification and characterization of three *Drosophila* MCMs, MCM2, MCM4, and MCM5, have been described (Treisman et al., 1995; Feger et al., 1995; Su et al., 1996, 1997). Here, we first describe their localization during embryonic divisions as detected by immunostaining of fixed *Drosophila* embryos. Fig. 1, A and B, shows nuclear staining for DmMCM2 during G₂ of cycle 14, dispersal of staining during mitosis 14, and reaccumulation in telophase 14 (DmMCM4 and DmMCM5 staining was indistinguishable from DmMCM2; not shown). Although all interphase cells exhibited nuclear MCM staining (see below), these early cell cycles lack a G₁ phase. Fig. 4 documents that DmMCM5 is nuclear during G₁ of cell cycle 17 (similar observations were made for DmMCM2 and DmMCM4; not shown). We conclude that these proteins are nuclear in G₁, S, and G₂, and that they are dispersed from chromatin upon entry into mitosis. This cell cycle pattern of localization is similar to the behavior of vertebrate MCMs (reviewed in Chong et al., 1996).

During the embryonic cycles that lack a G₁ phase, relocalization of MCMs to the nuclear region occurred as nuclei exit mitosis (Fig. 1). Association was first detected on the mitotic chromosomes at late anaphase/telophase (Fig. 1, C–H, white arrows). Relocalization of MCMs precedes and overlaps that of nuclear pore complexes (NPCs), as visualized by staining with WGA (Fig. 1, D and G; Finlay et al., 1987; see also Stafstrom and Staehelin, 1984). Therefore, this relocalization, at least initially, most likely results from binding of MCMs to chromatin and not to nuclear import. Similarly, localization of MCMs to chromatin precedes complete nuclear reformation in *Xenopus* (Coue et al., 1996). In both syncytial and postblastoderm cycles, nuclear MCM immunofluorescence increased further as cells progressed into interphase (e.g., Fig. 1, F–H, black arrows). This increase is possibly caused by import through NPCs: While only some MCMs contain sequences with obvious similarity to nuclear localization signals, they form complexes with each other, and analyses in *Xenopus* have documented import into intact nuclei (Madine et al., 1995a).

Cyclin Degradation and MCM–Chromosome Association

Next, we asked what mitotic event brings about chromosome association of MCMs. Chromosome association of MCMs correlates temporally with progression from metaphase to telophase. Progression beyond metaphase in many organisms is driven by proteolysis, the substrates of which include mitotic cyclins. Possibly, it is the degradation of one or more of these proteins that allows MCMs to associate with chromosomes. To ask if mitotic cyclins are involved, we analyzed chromatin association of MCMs when cyclin degradation is prevented.

Deletion of the “destruction box” at the amino termini of mitotic cyclins results in their stabilization in many systems, including *Drosophila* (Ghiara et al., 1991; Holloway et al., 1993; Surana et al., 1993; Sigrist et al., 1995). Express-

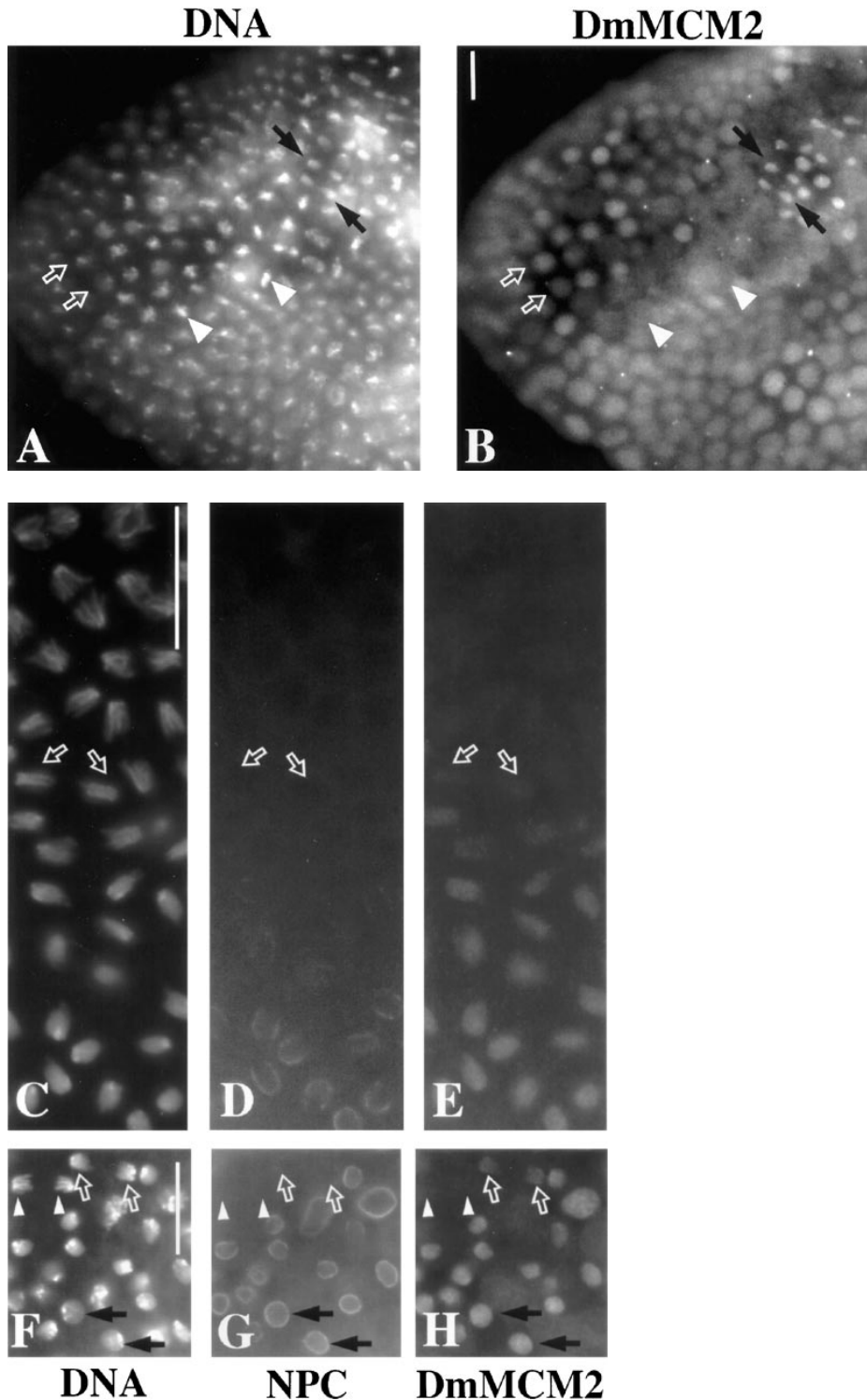


Figure 1. Subcellular localization of DmMCMs during embryonic divisions. *Drosophila* embryos were fixed and stained with DmMCM2 antibody (A, E, and H), WGA (to detect NPCs; D and G) and Hoechst 33258 (to visualize DNA; B, C, and F). (A and B) The head region of an embryo in transition from postblastoderm division cycle 14 to cycle 15 is shown. DmMCM2 antigen is present in the nuclei of G₂ cells in cycle 14 (white, open arrows). As cells enter mitosis, the chromatin condenses, and DmMCM2 antigen disperses from the nuclei into the cytoplasm (white arrowheads). DmMCM2 staining is again nuclear in anaphase/telophase cells (black arrows, see also C–H). (C–H) Reaccumulation of DmMCM2 to the nucleus at exit from mitosis during precellular nuclear division cycles (C–E) and postblastoderm cellular divisions (cycles 15; F–H). The embryo in C–E was undergoing a wave of mitoses such that nuclei toward the bottom of the figure are at a more advanced state of mitosis. A progression from anaphase (top) to telophase (bottom) is shown. In F–H, asynchronously dividing cells of a mitotic domain in M15 are shown. Chromosomes early in anaphase do not accumulate detectable levels of DmMCM2 (C–E, top, F–H, arrowheads). Reaccumulation of DmMCM2 (E and H) to the nuclear region begins in late anaphase (white, open arrows) and precedes the reappearance of WGA staining (D and G). Nuclear MCM stain increased as cells progressed into interphase (S phase of cycle 16) and completely reformed a nuclear envelope (F–H, black arrows). Bar, 20 μ m.

sion of stable cyclins from heat-inducible promoters and the consequences during postblastoderm embryonic divisions of *Drosophila* have been described (Sigrist et al., 1995). Similar experiments using our stable cyclin constructs reproduced these observations (Sprenger et al., 1997; see Materials and Methods). To restate briefly, in the presence of stable cyclins, cells enter mitosis but they arrest with condensed chromosomes. In a mitotic arrest by stable

cyclin A (cyclin A^s), the chromosomes and spindle have metaphase appearance and the sister chromatids remain together. In contrast, in an arrest caused by stable cyclin B (cyclin B^s) or coproduction of cyclins A^s and B^s, sister chromatids are separated as they would be at the metaphase–anaphase transition. Importantly, endogenous cyclins are degraded normally in an arrest caused by stable cyclins; i.e., cyclin B is degraded in an arrest caused by cy-

clin A^s, and cyclin A is degraded in an arrest caused by cyclin B^s (Sigrist et al., 1995).

To test if MCM–chromosome association requires cyclin degradation, we analyzed three *Drosophila* MCMs after induction of stable cyclins in cell cycles 14 and 15. All three MCMs dispersed from the nucleus upon entry to mitosis in the presence of stable cyclins, as they do in normal mitoses (not shown). During arrests in M14 and M15, reassociation with chromosomes occurred in the presence of cyclin B^s but not cyclin A^s (Fig. 2; data not shown). In the presence of cyclin A^s, DmMCM2 staining was uniform throughout the cell, except for exclusion from the region occupied by chromosomes (Fig. 2, panel 2, *arrowheads*). This is similar to what is seen in metaphases of wild-type embryos and heat-shocked w⁶⁷ controls lacking the transgenes (not shown). In contrast, in the presence of cyclin B^s, no such clearing of the DmMCM2 stain from the chromosomal region was seen. In most cells, colocalization of DmMCM2 stain and DNA stain is observed, suggesting concentration of DmMCM2 on chromosomes; this is similar to chromosomes in late anaphase of wild-type mitoses (e.g., Fig. 1, *F–H*, *white arrows*). The intensity of DmMCM2 stain on the chromosomes varied from cell to cell; this may be because cells of a *Drosophila* embryo undergo M14 and M15 asynchronously (Foe, 1989) and had been arrested in mitosis for various lengths of time at fixation.

While we cannot rule out that the lack of DmMCM2 stain on chromosomes that we report here and in Fig. 1 is caused by epitope masking at certain stages of mitosis, this notion is not consistent with the strong cytoplasmic staining of DmMCM2 seen at the same stages (e.g., in A^s arrest in Fig. 2, panel 2).

These results indicate that cyclin A^s is able to prevent DmMCM2 association with chromosomes, while cyclin B^s lacks the ability to do so under our experimental conditions. Consistent with this idea, when cyclins A^s and B^s were coexpressed, DmMCM2 was not associated with chromosomes (Fig. 2, panel 3, *arrowheads*). Staining for DmMCM4 and DmMCM5 showed that binding of these MCMs paralleled that of DmMCM2 (data not shown).

MCM–Chromosome Association Is Delayed When Mitosis Is Followed by a Prolonged Gap Phase

Our data show that all requirements for MCM–chromosome association are met during a mitotic arrest with cyclin B^s during embryonic mitoses M14 and M15. These mitoses are followed immediately by S phase and no gap phase. Thus, many activities required for MCM–chromosome association and DNA replication are likely to be present at these times. Later, upon the completion of M16, many epidermal cells withdraw from the cell cycle for the remainder of embryogenesis and enter a prolonged gap phase (G₁17; Foe et al., 1993). When cells were arrested in M16 with cyclin B^s, DmMCM2 (Fig. 3 *D*) stain was excluded from the region occupied by chromosomes (Fig. 3 *C*). These data indicate that in a cyclin B^s arrest in M16, all requirements for MCM–chromosome association are not met, whereas they were met in cyclin B^s arrest in M14 or M15.

The difference between M16 and earlier mitoses is also evident in careful analysis of unperturbed embryos. We found that chromosomes in late anaphase/telophase of

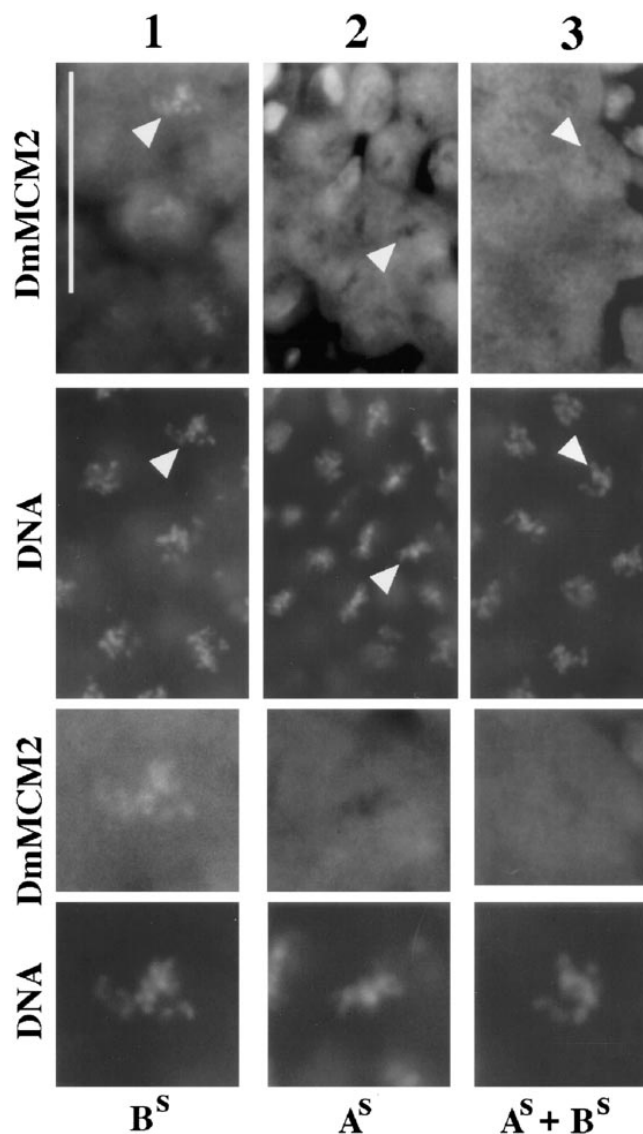


Figure 2. The effect of stable cyclins on association of DmMCM2 with chromosomes in mitosis. Embryos carrying heat-inducible transgenes for cyclin A^s (A^s), cyclin B^s (B^s), or both (A^s + B^s) were fixed and stained for DmMCM2 and DNA after heat induction. Cells indicated by arrowheads in the top two panels are magnified two times and shown in the bottom two panels. Note that regions occupied by chromosomes in panels 2 and 3 are devoid of DmMCM2 stain. The local exclusion of the stain is more obvious in a cyclin A^s arrest than in an arrest caused by coexpression of cyclins A^s and B^s, perhaps because the chromosomes are more tightly clustered in the former case. In contrast, cells arrested with cyclin B^s (panel 1) showed no exclusion of the stain from chromosomes, and in many cells, MCM stain on the chromosomes substantially exceeds the cytoplasmic signal (panel 1, *arrowheads*). Bar, 20 μ m.

M16 did not accumulate MCMs, whereas chromosomes at the same stage in earlier mitoses had acquired the MCM stain (compare bracketed nuclei in Fig. 4 *B* to those in Fig. 4 *A*). We conclude that chromosome association of MCMs does not occur or is greatly delayed in M16. One possible basis for the difference we have observed between M16 and earlier mitoses is the presence of an inhibitor of

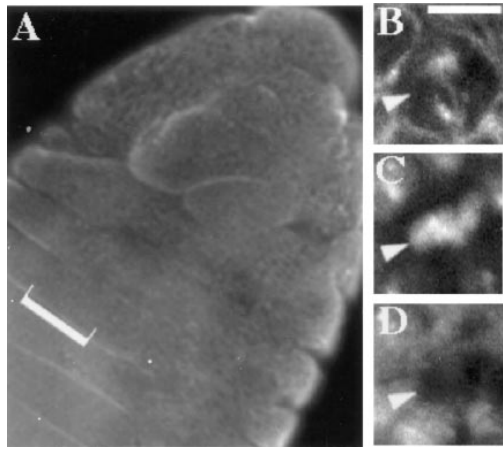


Figure 3. Chromosomes do not associate with DmMCM2 in a stable cyclin B arrest during M16. Embryos carrying the heat-inducible transgene for cyclin B^s were heat shocked, fixed after a rest period at room temperature, and stained for β -tubulin (B), DNA (C), and DmMCM2 (D). At this stage in embryogenesis (stage 14/15 in Campos-Ortega and Hartenstein, 1985), epidermal cells had ceased to divide in wild-type embryos. Therefore, cells blocked in M16 were identified by β -tubulin staining of mitotic spindles. A mitotic cell from the area indicated by the arrowhead in A (third thoracic segment) is shown at a higher magnification in B–D. Note the exclusion of DmMCM2 stain by chromosomes (C and D, arrowheads). Bar, 5 μ m in B–D.

MCM–chromosome association at this terminal mitosis. The data described below are consistent with this idea. Note, however, that once cells finish M16 and enter the next interphase, nuclear MCM staining is evident, presumably as a result of nuclear import (Fig. 4 C; see also Fig. 1, F–H, and text).

We also examined embryos arrested in M16 because of production of cyclin A^s or coproduction of cyclins A^s and B^s, or because of a mutation in *fizzy*, which is required for mitotic degradation of cyclins (Sigrist et al., 1995). We did not see chromosome association of DmMCM2 in any of these cases (data not shown).

Dap Inhibits MCM–chromosome association

Analysis of *dacapo* (*dap*) mutants suggests that this gene has a role in MCM–chromosome association in M16. *dap* encodes a p21/p27 type cdk inhibitor (Dap) whose developmentally regulated expression just before M16 contributes to the withdrawal of epidermal cells from the cell cycle (de Nooij et al., 1997; Lane et al., 1997). In homozygous *dap* mutants, many epidermal cells enter an additional S phase (S17) immediately after M16. Analysis of DmMCM2 in *dap* homozygotes showed that many late anaphase/telophase chromosomes acquire DmMCM2 in M16, unlike those in M16 of wild-type or heterozygous sibling embryos (Fig. 5). This is illustrated most clearly in embryos in which the DNA has been stained with the fluorescent dye Hoechst 33258 and DmMCM2 has been stained immunohistochemically. When the MCMs colocalize with DNA, the histochemical stain efficiently quenches the fluorescent staining (e.g., see Fig. 4). Focusing on the bright cells, one can see that the later stages of mitosis are not

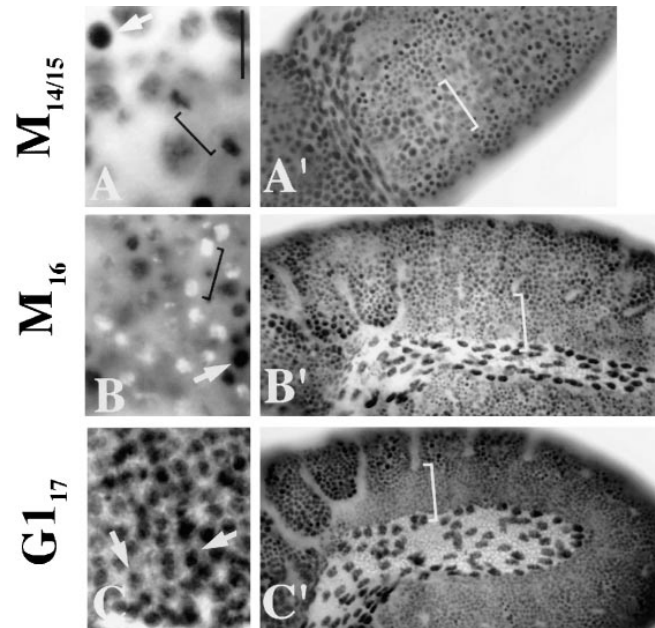


Figure 4. Chromosomes do not associate with DmMCM5 in M16 of wild-type embryos. Embryos were fixed and stained with purified antibodies against DmMCM5 and Hoechst 33258 to visualize DNA. The primary antibody was detected immunohistochemically for increased sensitivity (see Materials and Methods). Embryos were visualized simultaneously for DmMCM5 (in bright field) and DNA (fluorescence). DmMCM5 signal appears as a dark stain and, when it colocalizes with DNA, quenches the DNA fluorescence. All interphase nuclei are dark reflecting nuclear localization of MCMs in interphase (white arrows). (A and B) During cycle 15, DmMCM5 disperses from the nucleus in mitosis and reaccumulates on pairs of late anaphase/telophase chromosomes (A, bracket). Further reaccumulation is seen as nuclei progress into interphase (A, arrow). This pattern of DmMCM5 relocalization is identical to the pattern detected by immunofluorescence in Fig. 1, F–H. In contrast, cells of the dorsal epidermis that are going through M16 in a stage 11 embryo fail to acquire DmMCM5 stain at a similar stage in mitosis (B, bracket). Note, however, that when these cells have completed M16 and are in G1/17, DmMCM5 stain is present in the nuclei (B and C, arrows), presumably because of nuclear import (see Fig. 1 legend). A–C are magnified from areas indicated by brackets in A', B', and C'. Embryos are shown anterior to the right and dorsal facing forward (A') or down (B' and C'). Bar, 10 μ m in A–C.

quenched in cell cycle 16 of wild-type or heterozygous *dap* embryos, but they are quenched in homozygous *dap* mutant embryos (Fig. 5). For example, late anaphase/telophase nuclei in wild type/heterozygotes are devoid of histochemical DmMCM2 stain and are clearly visible when visualized for DNA (Fig. 5 A, boxes, and panels 1–3). In contrast, late anaphase/telophase nuclei in homozygous *dap* mutants have acquired the DmMCM2 stain and are barely visible when visualized for DNA (Fig. 5 B and panels 4–6). Not all anaphase/telophase figures acquire MCM stain in *dap* mutants (e.g., Fig. 5 B, arrowhead). Likewise, not all cells of the epidermis undergo S17 in a homozygous *dap* mutant (de Nooij et al., 1997; Lane et al., 1997). Direct analysis of DmMCM2 immunofluorescent staining also revealed association of MCMs with late anaphase/telophase nuclei of homozygous *dap* mutants (not shown). These

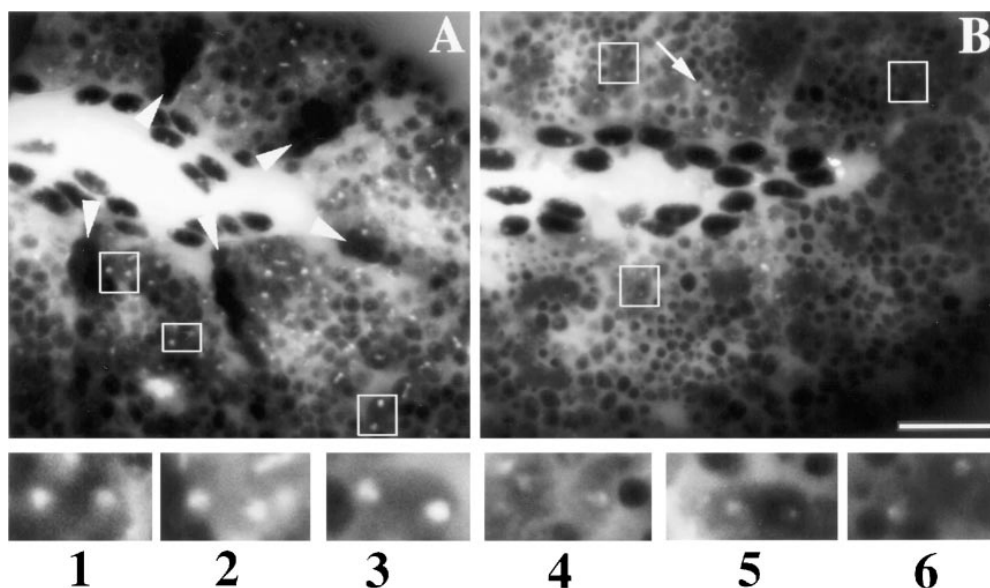


Figure 5. MCM–chromosome association in M16 in *dacapo* mutants is detected by histochemistry. Homozygous *dap* mutant embryos and their wild-type or heterozygous siblings were fixed and stained for DmMCM2 and DNA. Histochemical staining used for detection of DmMCM2 results in a dark-colored deposit that quenches the DNA stain when DmMCM2 and DNA are colocalized. Lateral views of the posterior third of stage 11 embryos are shown in *A* and *B*. Many of the epidermal cells in this view are completing M16 (dorsolateral epidermis). (*A*) In wild type or heterozygotes, identified by β -galactosidase stain in the *wingless*

pattern (arrowheads), many pairs of anaphase/telophase chromosomes lack the DmMCM2 stain and are seen as brightly fluorescent (boxes show examples magnified in panels 1–3). This is similar to what is seen for DmMCM5 in wild-type embryos undergoing M16 (Fig. 4 *B*). (*B*) In contrast, in *dap* homozygous mutants, identified by the absence of β -galactosidase stain, many anaphase/telophase chromosomes acquire DmMCM2 stain, which quenches the DNA signal. Consequently, chromosome pairs (boxes) are not readily visible, except when magnified as in panels 4–6. Bar, 20 μ m in *A* and *B*; 7 μ m in panels 1–6.

data suggest that expression of Dap in cycle 16 normally inhibits chromosome association of DmMCM2.

A summary of data on MCM–chromosome interactions is shown schematically in Fig. 6 and described below.

Discussion

The normal cycle of association and dissociation of MCM proteins from chromatin is thought to play a role in the cell cycle oscillations in the competence of DNA for replication. To investigate whether the mitotic association of *Drosophila* MCMs to chromatin is controlled by the destruction of cyclins, we have analyzed the ability of stabilized cyclins to block this association. Our results show that stable cyclin A, but not stable cyclin B, can block this mitotic association. Additionally, a difference in the behavior of MCMs during mitoses that are followed immediately by S phase (M14 and M15) and a mitosis that is followed by a prolonged G₁ quiescence (M16) indicates that MCM association may require other factors in addition to the destruction of mitotic cyclins.

The association of MCM proteins to condensed chromosomes in M14 and M15 arrested with stable cyclin B demonstrates that this association requires neither decondensation of the chromosomes nor assembly of a nuclear membrane. This finding is consistent with the observed kinetics of association of MCMs with chromosomes upon exit from mitosis in unperturbed cycles of vertebrates (Todorov et al., 1994; Coue et al., 1996; Schulte et al., 1996) and *Drosophila* (this report). The ability of stable cyclin A to block MCM association to mitotic chromosomes suggests either that the normal cyclin A can inhibit this association or that the deletion of the destruction box

and flanking sequences produced a novel activity. While we can not presently eliminate the latter alternative, we suggest that inhibition of MCM association to chromatin by cyclin A:cdc2 kinase might contribute to the failure of MCMs to associate with chromosomes during metaphase. The activation of the proteolytic machinery and destruction of cyclin A, and possibly other inhibitors of MCM–chromosome association, would then time the beginning of the assembly of replication proteins onto DNA.

How might cyclins or cyclin-dependent kinases control MCM behavior? Some members of the MCM family contain putative cdk phosphorylation sites. In *Xenopus* extracts, the phosphorylation state of a MCM homologue, XMCM4, has been shown to change in concert with mitotic kinase activity (Coue et al., 1996), and cdks can direct phosphorylation of XMCM4 and promote its dispersal from the nucleus in vitro (Hendrickson et al., 1996). Direct inhibition of MCM–chromosome association by mitotic cyclin/cdk phosphorylation is suggested by these analyses. However, our efforts to detect differential modification of MCMs on the basis of mobility on denaturing and native gels have been negative (Su, T.T., and P.H. O’Farrell, unpublished data). In addition, we cannot exclude more indirect paths of regulation. For example, association of *Xenopus* MCMs to chromatin requires CDC6 and ORC2 (Coleman et al., 1996). Thus, cdks could influence the activity of these proteins and thereby affect MCM–chromosome association indirectly.

A differential ability of *Drosophila* cyclins A and B to block MCM–chromosome association might be relevant to previous findings that cyclin A, but not cyclin B, prevents rereplication of DNA in G₂ in *Drosophila*. That is, rereplication in cyclin A mutants occurs in the presence of cyclin

B (Lehner and O'Farrell, 1990; Sauer et al., 1995). Perhaps inhibition of MCM–chromosome association by cyclin A contributes to the block to rereplication.

In many ways, our results parallel recent findings in *Xenopus* extracts (Mahbubani et al., 1997) in which MCMs have been shown to contribute to replication licensing factor (RLF) activity (Blow and Laskey, 1988; Chong et al., 1995; Madine et al., 1995a). (a) RLF becomes active at metaphase–anaphase transition; *Drosophila* MCMs binding to chromosomes follows metaphase–anaphase transition. (b) A cdk-dependent activity renders RLF inactive in metaphase; cyclin A appears to prevent chromosome association of *Drosophila* MCMs, presumably until its degradation. (c) 6-Dimethylaminopurine, which prevents RLF activation, has been shown to stabilize a mitotic cyclin; we show here that stabilization of a mitotic cyclin prevents MCM–chromosome association. Thus, our results complement the biochemical data in *Xenopus* and, additionally, provide evidence for the role of mitotic kinases in regulation of MCM behavior in vivo.

Before cycle 16 of the embryo, each mitosis is followed immediately by S phase. We suggest that any necessary preparation for DNA synthesis must occur during mitosis in these cycles to allow immediate progression into S phase subsequently. Accordingly, we found that binding of MCMs to chromosomes occurs during mitosis in precycle 16 mitoses we have examined. In contrast, M16 is followed by a long gap phase in epidermal cells. Thus, at this stage in embryogenesis, there is an extended postmitotic period during which cells might prepare for DNA synthesis and binding of MCMs to chromosomes might occur at any time before S phase. Indeed, at this stage, we find that MCMs do not bind chromosomes in M16.

The change in MCM–chromosome association seen at M16 is unlikely to be a direct outcome of the transition from maternal to zygotic control of MCM transcription. This is because in *Drosophila* embryos, maternal–zygotic transition occurs earlier, during cycle 14 (Foe et al., 1993). The switch from maternal to zygotic transcription of DmMCM2, DmMCM4, and DmMCM5 also occurs at this time (Treisman et al., 1995; Su, T.T., and P.H. O'Farrell, unpublished observations). Furthermore, mutants in MCM genes show no defect until later stages, indicating that the maternal supply of MCM gene products is sufficient for

these early stages we have examined (Feger et al., 1995; Treisman et al., 1995).

While the result in M16 appears to contradict reports of MCM–chromosome association during mitosis in mammalian cell cycles that contain a G_1 , we wish to stress that G_1 17 in *Drosophila* epidermal cells lasts for the rest of embryogenesis and terminates only upon hatching of the larvae. Thus, G_1 17 may be different from G_1 of cycling cells and resemble a quiescent state. Indeed, although MCMs appear to be chromatin-bound in G_1 of cycling mammalian cells, they are downregulated in G_0 cells (Schulte et al., 1996).

We conclude that, unlike in earlier mitoses, all requirements for chromosome association of MCMs are not met in M16. Similarly, in an arrest with cyclin B^s during M16, DmMCM2 was not present on chromosomes, unlike in cyclin B^s arrests at earlier mitoses. The inability of M16 chromosomes to bind MCMs appears to depend on *dap*, which is expressed for the first time in G_2 16 in epidermal cells. In *dap* mutants, DmMCM2 binds chromosomes in M16 (this report) and cells enter S phase instead of G_1 17 (de Nooij et al., 1997; Lane et al., 1997). Our data, therefore, implicate Dap as an inhibitor of chromosome association of MCMs.

Dap is a specific inhibitor of cyclin E:cdk2 complexes, and its role in promoting the arrest of cycle 17 cells in G_1 is thought to result from its contribution to the downregulation of cyclin E (Lane et al., 1997). Indeed, downregulation of cyclin E is known to be essential for G_1 quiescence in cycle 17, and expression of cyclin E is sufficient to drive ectopic S phase (Knoblich et al., 1994). This raises the possibility that Dap inhibits MCM–chromosome association by inhibiting cyclin E:cdk2. Perhaps cyclin E:cdk2 promotes chromosome association of MCMs. The behavior of MCMs in mitoses up to and including M16 is consistent with this idea (Fig. 6). First, cyclin E is present continuously in embryonic cycles in which S follows M (Knoblich et al., 1994); MCMs associate with mitotic chromosomes after metaphase in these cycles (Fig. 6A). Second, cyclin E activity is absent in M16, which is followed not by S but by G_1 17 (Knoblich et al., 1994); likewise, MCMs do not associate with M16 chromosomes (Fig. 6B). In *dap* mutants, M16 is followed by S17, and cyclin E activity is thought to persist (Fig. 6C). On the basis of these data, we propose

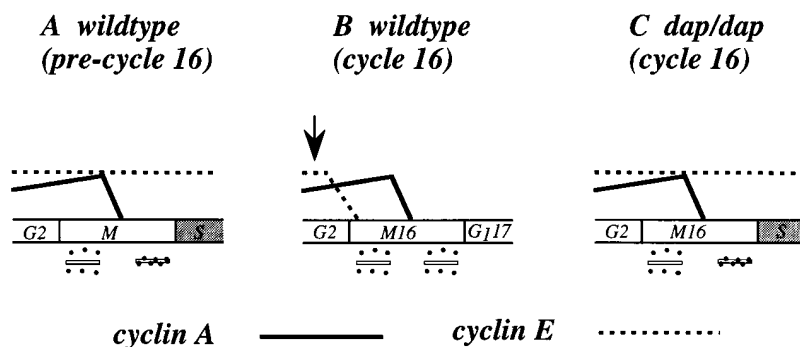


Figure 6. A summary of cyclin profiles and MCM–chromosome association. (A) In wild-type embryonic cycles in which S phase follows M, cyclin E (dashed line) is present continuously, and association of MCMs (dots) with chromosomes (bars) occurs as mitotic cyclins (solid lines) are degraded during mitosis. (B) In M16, cyclin E–associated activity is down regulated as *dacapo* is expressed (arrow), and MCMs fail to associate with chromosomes, even when mitotic cyclins are degraded (Lehner and O'Farrell, 1989). (C) In *dap* mutants, cyclin E–associated activity persists, MCMs associate with chromosomes as described in A, and M16 is followed by

S phase. We propose that cyclin:cdk activity is coupled to MCM–chromosome association in the following manner: a cyclin A–associated activity inhibits chromosome association of MCMs. After the loss of cyclin A in mitosis, chromosome association of MCMs requires a second cyclin-dependent activity that is provided by cyclin E:cdk2. The diagrams are not drawn to scale.

that mitotic cyclin degradation creates a permissive state for MCMs to associate with chromosomes, but that cyclin E:cdk2 also contributes positively to this association. Observations in mammalian cultured cells suggests that mitotic association of MCMs and chromosomes can occur in cell cycles that include a G₁ and lack cyclin E during mitosis (Schulte et al., 1996; Sherr, 1994). We do not know the basis for these different behaviors, but we suggest that the activity provided by cyclin E in *Drosophila* may be supplied otherwise, perhaps by a redundant function, in mammalian cells.

In contrast to our proposal that cyclin E:cdk2 stimulates MCM–chromosome association, a recent report argued that addition of high levels of cyclin E to *Xenopus* extracts can inhibit MCM binding to sperm chromatin (Hua et al., 1997). However, this report emphasized the role of cyclin E in the inhibition of DNA rereplication. We concur with this emphasis and argue that cyclins make both positive and negative inputs to DNA replication and the assembly of proteins (such as MCMs) at origins. In *Drosophila*, both cyclins A and E are capable of driving G₁ cells into S phase, yet both cyclins A and E are able to inhibit postreplicative cells from rereplicating their DNA (Sauer et al., 1995; Sprenger et al., 1997; Follette, P.J., R.J. Duronio and P.H. O'Farrell, manuscript in preparation; reviewed in Su et al., 1995, and Wuarin and Nurse, 1996). In yeast, cyclins have both stimulatory and inhibitory inputs into DNA replication (reviewed in Diffley, 1996; Nasmyth, 1996). In *Xenopus*, depletion of the endogenous cyclin E demonstrates that replication in *Xenopus* extracts requires cyclin E (Fang and Newport, 1991; Jackson et al., 1995), yet high levels of exogenous cyclin E block the binding of MCM protein to newly added chromatin (Hua et al., 1997). These observations are consistent with the idea that cdk activity has at least two inputs into cycles of replication, one positive (perhaps leading to binding of MCMs to chromatin) and one negative (possibly inhibiting rebinding of MCMs to chromatin).

In conclusion, our data suggest a role for mitotic cyclin degradation in chromosome binding of replication factors, MCMs. Degradation of mitotic cyclins is essential for the completion of mitosis. The completion of mitosis thus appears to be intimately linked to preparation for DNA replication by a common event, degradation of mitotic cyclins. As a result, the two major phases of the cell cycle, mitosis and DNA replication, may be viewed, not as discrete phases that must be ordered, but rather, as events that are coupled such that completion of one may occur concurrently with preparation for the other.

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